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The Role of the AcMNPV *25K* Gene, "*FP25*," in Baculovirus *polh* and *p10* Expression

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A previous study showed that an *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) *25K* mutant produced less polyhedrin protein than wild-type (Jarvis *et al., J. Virol.* 66, 6903–6911, 1992). In this study, the role of the *25K* gene product (AcMNPV ORF 61) in baculovirus gene expression was further investigated. Five different viral *25K* mutants expressed lower levels of polyhedrin protein and less CAT activity under the control of the *polh* promoter compared to wild-type. *Polh* RNA was equally stable in wild-type and mutant virus-infected cells while the rate of *polh* transcription was significantly reduced in mutant-infected cells. In comparison, steady-state levels of *p10* RNA were not reduced in *25K* mutant-infected cells, indicating that the reduction in *polh* RNA did not reflect a general effect on very late gene transcription. Expression of *ie-1,* which also appears to influence *polh* expression (Choi and Guarino, *Virology* 209, 90–98, 1995), was not influenced by *25K* mutation. These results show that the 25K protein is important for maintaining optimal levels of *polh* transcription by a mechanism that does not involve maintaining *ie-1* expression.

INTRODUCTION

Mutation of the *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) *25K* gene results in altered assembly of the occluded form of baculoviruses. These alterations, which include a reduced level of viral occlusion production, the assembly of occlusions that lack virions, aberrations in occlusion-derived virus (ODV) envelopment, and an increase in budded virus (BV) production (Fraser, 1986, 1987; Harrison and Summers, 1995a), are collectively referred to as the "few polyhedra" or FP phenotype. The AcMNPV *25K* gene encodes a 25-kDa protein which is present in the cytoplasm in association with amorphous cytoplasmic masses and also in association with electron-dense structures located at the periphery of the nucleus of infected cells (Harrison and Summers, 1995b). In a study of the nuclear localization of the baculovirus polyhedrin protein, Jarvis *et al.* (1992) reported that the rates of both polyhedrin nuclear localization and polyhedrin biosynthesis were reduced in cells infected with the *25K* mutant called AcFPβgal. This result indicated that the *25K* protein exerts some influence on the expression of the polyhedrin (*polh*) gene.

During infection of susceptible host cells, baculovirus genes are expressed in four sequentially ordered, temporally distinct phases (immediate early, delayed early, late, and very late). Two baculovirus genes, *polh* and *p10,* are expressed at remarkably high levels during the very late phase of infection (Rohel *et al.,* 1983; Smith *et al.,* 1983). *Polh* encodes polyhedrin, the major structural component of viral occlusions (Vlak and Rohrmann, 1985; Rohrmann, 1986). *P10* encodes a 10-kDa protein associated with cytoplasmic and nuclear fibrillar structures in infected cells (van der Wilk *et al.,* 1987). Both *polh* and *p10* are nonessential for baculovirus replication in cultured cells, and the promoters from these genes are used to drive high-level foreign gene expression by baculovirus expression vectors (Luckow and Summers, 1988b; Vlak *et al.,* 1990; O'Reilly *et al.,* 1992).

Both transient expression assays and temperature-sensitive mutant viruses have been used to identify many factors that influence expression of the very late genes. Transient expression assays have identified 18 genes of AcMNPV that are required for expression of a reporter gene under the control of the promoters for both the late gene, *vp39* (the gene encoding the major capsid protein; Thiem and Miller, 1989), and *polh* (Passarelli and Miller, 1993; Todd *et al.,* 1995). Additionally, McLachlin and Miller (1994) identified a gene (very late expression factor-1 or *vlf-1*) required for the strong expression of very late genes. A virus bearing a point mutation in this gene (*ts*B837) exhibited a temperature-sensitive phenotype in which very late gene expression was reduced but late gene expression was unaffected. More recently, Todd *et al.* (1996) reported that *vlf-1* was required for optimal expression from very late promoters in a transient expression assay. In a study of a temperature-sensitive mutant virus bearing point mutations in the gene encoding the immediate early-1 (*IE-1*) transregulatory protein (Guarino and Summers, 1986a; Ribiero *et al.,* 1994), Choi and Guarino (1995) also found that polyhedrin synthesis was reduced when cells infected with this virus were

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shifted to the nonpermissive temperature. The expression levels of two other proteins were unaffected under these conditions, suggesting that IE-1, which has been well characterized as an activator of early gene expression, might also participate directly in very late gene expression.

The observation that the 25K protein does not seem be directly involved in the assembly events affected by *25K* mutation suggests that it may influence such events indirectly, possibly by way of modulating the expression of other viral genes. Also, the finding that *25K* influences normal levels of polyhedrin biosynthesis suggests that it, too, might encode a very late gene expression factor. Hence, in this study, the influence of *25K* on *polh, p10,* and *ie-1* expression was investigated. Overall, the results showed that *25K* is required for normal rates of *polh* transcription. *P10* RNA levels were unaffected by *25K* mutation, a finding that supports other studies suggesting that *polh* and *p10* are regulated by apparently different mechanisms (Roelvink *et al.,* 1992; van Oers *et al.,* 1992; Chaabihi *et al.,* 1993). IE-1 protein synthesis and *ie-1* promoter-driven reporter gene expression were unaffected by *25K* mutation, suggesting that the influence of *25K* on *polh* expression does not result from an indirect effect on *ie-1* expression. The implications of these results for very late gene expression and the relationship between these effects and the FP phenotype are discussed.

MATERIALS AND METHODS

Cells and virus

Sf9 cells (cloned from the *Spodoptera frugiperda* IPLB-Sf21-AE cell line, Vaughn *et al.,* 1977) were grown in TNM-FH medium containing 10% heat-inactivated fetal calf serum (JRH Biosciences, Lenexa, KS), amphotericin B, gentamycin, and 0.1% (w/v) pluronic F68 (BASF Wynandotte Corp., Parsippany, NJ; Murhammer and Goochee, 1988). The AcMNPV strain E2 (Summers and Smith, 1987) was used as wild-type virus. The FP mutants AcFP480-1, AcFP480-2, AcFP875-1, AcFP875-2, and AcFPβgal have been described previously (Beames and Summers, 1988, 1989; Harrison and Summers, 1995a; see Fig. 1). Briefly, AcFP480-1 and AcFP875-1 contain deletions within the *25K* open reading frame (ORF). AcFP480-2 contains an insertion of a putative host transposable element in the *25K* ORF, and AcFP875-2 contains a host transposon insertion in the *25K* promoter region. AcFPβgal contains an in-frame *lacZ* insertion that interrupts the *25K* ORF and encodes a *25K*–β-galactosidase fusion protein. AcVL941-500βgal (Luckow and Summers, 1989) contains a nonfused form of β-galactosidase in place of the polyhedrin gene. Ac360-CAT contains a wild-type *25K* gene and a polyhedrin-chloramphenicol acetyltransferase (CAT) fusion gene in which the first 11 codons of *polh* are fused in-frame to CAT (Luckow and Summers, 1988a). AcFPβgal-CAT is essentially the AcFPβgal virus containing the same *polh*–CAT fusion gene that is present in Ac360CAT (Harrison and Summers, 1995a). Occlusion-positive viruses were titered by end-point dilution and occlusion-negative viruses were titered by plaque assay (Summers and Smith, 1987).

Construction of recombinant viruses

Recombinant viruses were constructed essentially as described by Summers and Smith (1987). For the construction of AcFP480-1-CAT, AcFP480-2-CAT, AcFP875-1-CAT, and AcFP875-2-CAT (Fig. 1), viral DNA from each parental FP virus stock was cotransfected with plasmid pVL775, which was used to construct the Ac360-CAT recombinant (Luckow and Summers, 1988a). Recombinant viruses arising by homologous recombination were identified by their occlusion-negative plaque phenotypes.

Recombinant E2 and FP viruses containing *ie-1*/CAT fusion genes in the *polh* locus also were constructed (Fig. 1). The CAT gene in these viruses is under the transcriptional control of the *ie-1* promoter region. The source of the CAT gene for these viruses was pCAT, a plasmid constructed by cloning the 775-bp CAT-containing *Bam*HI fragment from pVL775 into the *Bam*HI site of pUC18. A *Hin*cII–*Kpn*I fragment containing the CAT insert was excised from pCAT. The protruding 3′ end generated by *Kpn*I was removed by treatment of the fragment with T4 DNA polymerase. The fragment was then cloned by blunt-end ligation into pAcIE1 (Guarino and Summers, 1986a) at the *Hin*cII sites located at −39 and +1253 with respect to the *ie-1* translational initiation codon. This construct was designated p-39CAT. Another *ie-1*/CAT fusion construct was assembled using the plasmid pAcIE1600*Bam*HR (Jarvis *et al.,* 1996), a modified version of pAcIE1 in which most of the *ie-1* ORF has been replaced by a linker containing a *Bam*HI site immediately downstream of the *ie-1* translational initiation codon. This construct contains the entire *ie-1* 5′ untranslated region (UTR) as well as a copy of the *hr*5 enhancer (Guarino and Summers, 1986b) upsteam of the promoter region. The *Bam*HI CAT-containing insert from pCAT was cloned into pAcIE1600BamHR at the *Bam*HI site downstream of the native *ie-1* initiation ATG, producing an *ie-1*/CAT fusion that has a full-length *ie-1* 5′ UTR. This construct was designated pHR+1CAT. Transfer vectors designed to insert the *ie-1*/CAT fusions into the *polh* locus in the viral genome were prepared by digesting pVL1393 with *Sma*I and *Eco*RV to remove the *polh* promoter from the plasmid. The *ie-1*/CAT fusions were excised as *Sma*I–*Hin*dIII fragments from p-39CAT and pHR+1CAT and the 3′ recessed ends generated by *Hin*dIII digestion were filled in by treatment with Klenow fragment. The inserts were cloned by blunt-end ligation into the *Eco*RV and *Sma*I sites of pVL1393 in both orientations, producing a total of four different transfer vectors. Each transfer vector was

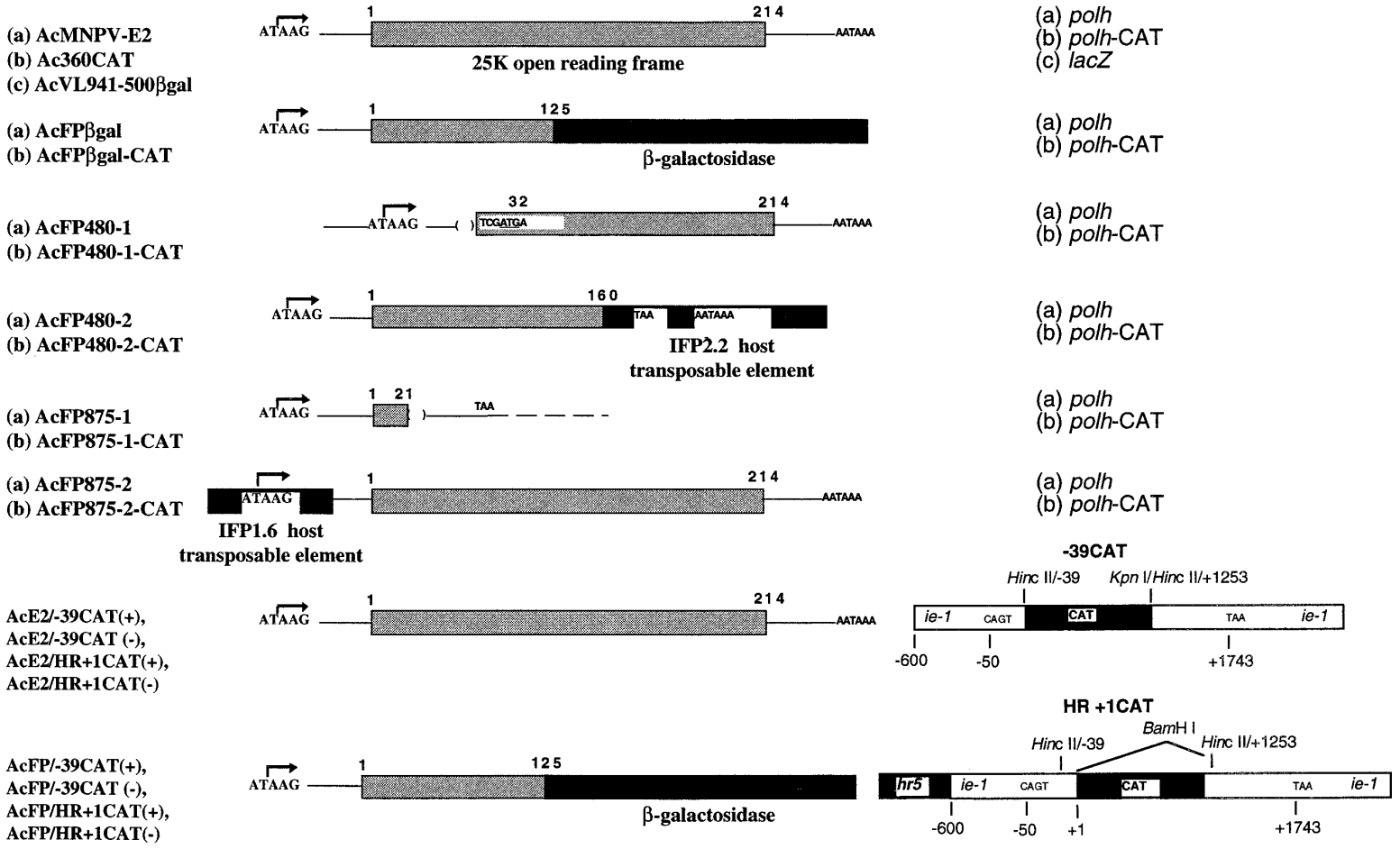

FIG. 1. Viruses used in this study. For each virus listed, a schematic representation of *25K* is presented and the gene inserted at the *polh* locus is indicated. *25K* coding sequences are represented by shaded bars, and transposable element and *lacZ* insertions are represented by black bars. Amino acid positions (numbers), termination codons (TAA), polyadenylation signals (AATAAA), the *25K* late gene transcription initiation site (ATAAG), and the in-frame initiation codon for the AcFP480-1 *25K* gene (TCGATGA) are indicated. Numbers indicate the nucleotide positions of restriction sites, polyadenylation signals, and the transcription initiation motif (CAGT) with respect to the *ie-1* translation initiation codon (+1) in the unaltered *ie-1* gene.

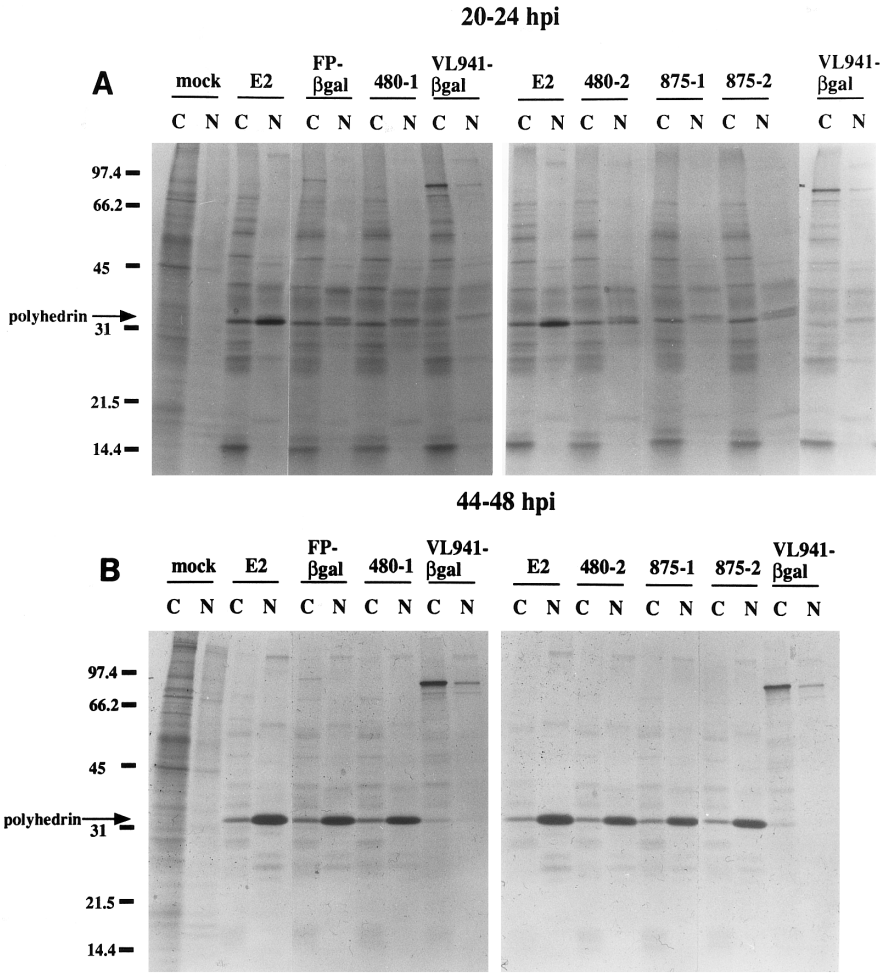

FIG. 2. Influence of *25K* on polyhedrin biosynthesis. Sf9 cells were infected with wild-type (E2) AcMNPV, various *25K* mutants (FPβgal, 480-1, 480-2, 875-1, 875-2), or a polyhedrin-negative recombinant virus (VI941-βgal) and radiolabeled from 20-24 (A) or 44-48 (B) hr p.i. with Translabel. After labeling, cytosolic and nuclear fractions were prepared and 5×10^4 cell equivalents of each fraction were solubilized and analyzed by SDS–PAGE and autoradiography. The molecular weight standards and the location of polyhedrin are indicated on the left.

cotransfected into Sf9 cells with AcMNPV-E2 or FPβgal viral DNA and recombinant viruses were identified and plaque-purified. The names of the resulting viruses were based on the parental virus stock, the *ie-1*/CAT fusion used to produce the recombinant, and the orientation of the fusion gene in the *polh* locus. For example, AcE2/-39CAT(−) is an E2-based recombinant containing the −39CAT fusion transcribed in the polyhedrin antisense (−) orientation, and AcFP/HR+1CAT(+) is an FPβgal-based recombinant containing the HR+1CAT fusion transcribed in the polyhedrin sense (+) orientation.

Biochemical fractionation

Sf9 cells were seeded into 25-cm^2 tissue culture flasks at a density of 3×10^6 cells/flask and infected with AcMNPV-E2, AcFPβgal, AcFP480-1, AcFP480-2, AcFP875-1, AcFP875-2, or AcVL941-500βgal at a multiplicity of infection (m.o.i.) of 50. Proteins were metabolically labeled from 20 to 24 or from 44 to 48 hr p.i. with 100 μCi/ml of Translabel (80% [^{35}S]methionine and 20% [^{35}S]cysteine; ICN Radiochemicals, Irvine, CA) in methionine-free Grace's medium. After labeling, the cells were separated into cytosolic and nuclear fractions by a previously described detergent-based fractionation procedure (Jarvis *et al.,* 1991).

Equivalent aliquots of both fractions were solubilized and analyzed by discontinous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Laemmli, 1970) on 12% polyacrylamide gels. The gels were stained with Coomassie blue R-250, dried, and subjected to autoradiography. This experiment was carried out twice to ensure reproducibility of the results.

CAT assays

Sf9 cells were seeded into 6-well plates at a density of 1×10^6 cells/well, infected with various CAT-expressing viruses at an m.o.i. of 5, and extracts were prepared at various times after infection as described by Guarino

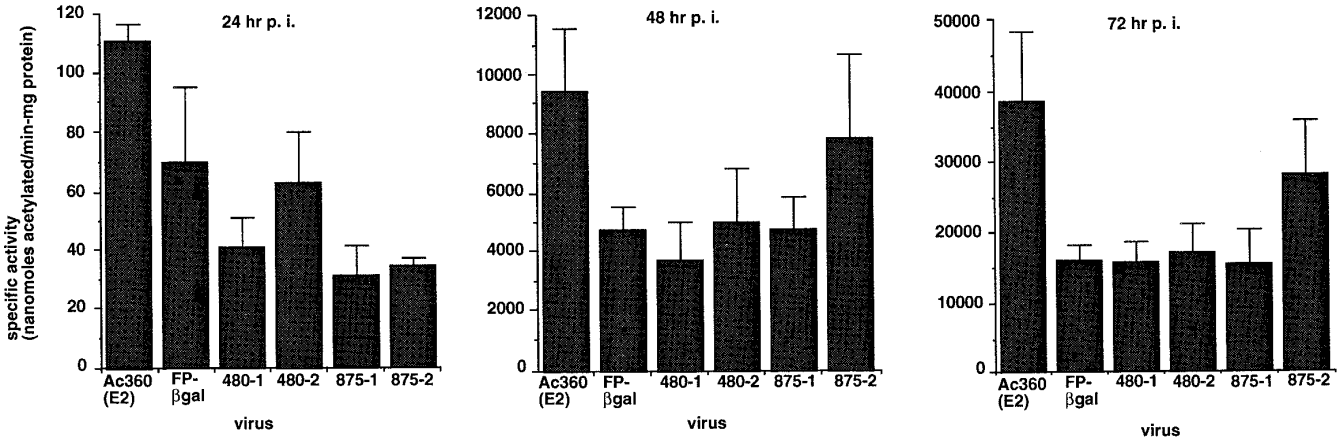

FIG. 3. Influence of *25K* on *polh* promoter-driven CAT gene expression. Sf9 cells were infected with recombinant baculoviruses containing a *polh*/CAT fusion gene in place of *polh* in a wild-type (Ac360) or mutant (FPβgal, 480-1, 480-2, 875-1, and 875-2) *25K* background. At 24, 48, and 72 hr p.i., infected cell extracts were prepared and assayed for CAT activity. The graphs show the average specific activity for three replicate samples/virus/time point. The error bars represent one standard deviation.

and Summers (1986a). CAT activity in the extracts was measured as described by Gorman *et al.* (1982) with the modifications of Guarino and Summers (1986a) using [^{14}C]chloramphenicol (DuPont NEN, Wilmington, DE) under conditions in which less than 30% acetylation was obtained. The protein content of each extract was measured by Bradford assay and used to calculate specific activities as nanomoles of chloramphenicol acetylated/min/mg protein.

Quantitative primer extension analysis

For all RNA analyses, Sf9 cells were seeded in 150-cm^2 flasks at a density of 1.8×10^7 cells per flask and infected with E2 or FP mutant viruses at an m.o.i. of 10. For measurement of steady-state RNA levels, cells were harvested at various time points and total RNA was prepared by the method of Chirgwin *et al.* (1979). All RNA analyses were carried out at least three times to ensure the reproducibility of the results.

The following oligonucleotides were used for primer extension analysis: polh-263, 5′-TTGTAGTACTTGTTGTC GTACACGTAGGTACGCCCGATGG-3′; cap-501, 5′-TTTGTCGCGGCGCCATACCCACGGGCACTAGCGCCA-TATTG-3′; and p10-407, 5′-CGTAACGGCGTCTAAAAT-TTGCGTCAAAACGTTAGGCTTTG-3′. Polh-263 extends from nt 65 to 26 in the *polh* sequence published by Hooft van Iddekinge *et al.* (1983). Cap-501 extends from nt 583 to 460 in the *vp39* sequence published by Thiem and Miller (1989). P10-407 extends from nt 45 to 5 in the *p10* sequence published by Kuzio *et al.* (1984). The oligonucleotides were purified by denaturing urea–polyacrylamide gel electrophoresis prior to use in primer extension assays (Ellington, 1989). The purified oligonucleotides were 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (DuPont NEN) by a standard protocol (Sambrook *et al.,* 1989). Labeled primer (200,000 cpm) was annealed to 10–30 μg of RNA. RNA/primer hybrids were ethanol-precipitated, washed in 70% ethanol, and resuspended in 30 μl of reverse transcriptase reaction mix [50 m*M* Tris–HCl (pH 7.6), 60 m*M* KCl, 10 m*M* $MgCl_2$, 0.66 m*M* dNTPs, 1 m*M* dithiothreitol (DTT), 40 U RNasin, 50 μg/ml actinomycin D, 150 U Moloney murine leukemia virus reverse transcriptase (United States Biochemical, Cleveland, OH)]. The annealed primers were extended at 42° for 2 hr, and then the reaction products were ethanol-precipitated and resuspended in 2 μl of 0.1 *N* NaOH. After a 30-min incubation to eliminate the RNA template, 4 μl of sequencing stop/loading buffer was added to the reaction products. The samples were boiled for 3 min and analyzed by electrophoresis on a 6% urea–polyacrylamide sequencing gel together with a sequencing ladder generated by the same oligonucleotides used for the primer extension. The gels were dried and subjected to autoradiography, and the primer extension products were quantitated with the FUJIX BAS2000 bio-imaging analyzer system (Fuji Photo Film Co., Japan).

Analysis of RNA stability

Sf9 cells were seeded into 150-cm^2 flasks and infected with AcMNPV-E2 or AcFPβgal at an m.o.i. of 10. At 24 hr p.i., the medium was removed and replaced with medium containing 200 μg/ml of cordycepin (3′-deoxyadenosine; Sigma Chemical Co., St. Louis, MO), with medium containing an equivalent concentration of ethanol, or with medium alone. Cytoplasmic RNA was isolated every 6 hr until 48 hr p.i. by the technique of Mainprize *et al.* (1986) with modifications. Briefly, infected cells were pipetted off the surface of the flasks and pelleted by low-speed centrifugation. The cells were then resuspended in TNM buffer [30 m*M* Tris–HCl (pH 7.6), 100 m*M* NaCl, 10 m*M* $MgCl_2$] containing 0.5% Nonidet-P40 (NP-40) and incubated on ice for 5 min with gentle shaking. The nuclei

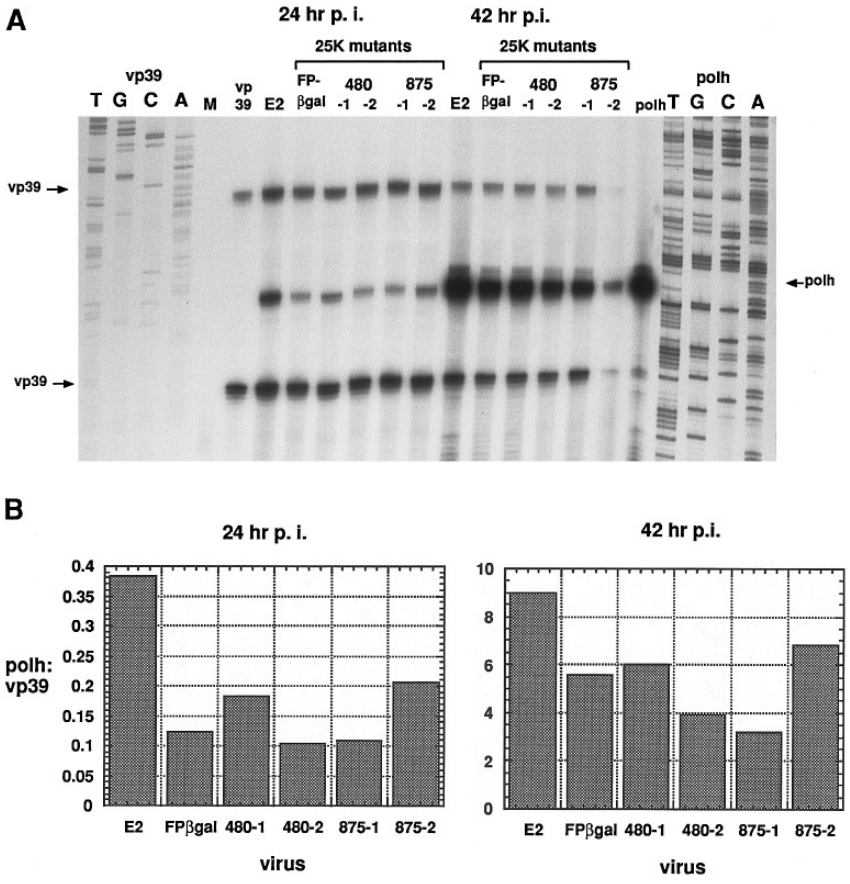

FIG. 4. Influence of *25K* on steady-state levels of *polh* RNA. (A) Total RNA from wild-type AcMNPV (E2)- or *25K* mutant-infected Sf9 cells was used for primer extensions with the *polh*- and *vp39*-specific primers. Control reactions were carried out with RNA from mock-infected cells (M) and with E2 RNA using the *polh*-specific or *vp39*-specific primers separately. Sequencing ladders generated with the *polh*-specific and *vp39*-specific oligonucleotides are shown to the right and left of the primer extension products, respectively, and the locations of the *vp39* and *polh* extension products are indicated. (B) The *vp39* and *polh* extension products were quantitated with the FUJIX BAS2000 bio-imaging system and corrected for background radiation in each lane. The ratios of *polh* to *vp39* RNA were calculated for each sample by dividing the amount of *polh* radioactivity by the total radioactivity in the two *vp39* bands in (A) and the ratios were graphed.

were pelleted by centrifugation for 6 min at 12,000 *g*. The supernatant was transferred to fresh microfuge tubes and extracted first with phenol and then with a mixture of 1 part phenol and 1 part chloroform:isoamyl alcohol (24:1), and finally with chloroform:isoamyl alcohol (24:1). The RNA was ethanol-precipitated, resuspended in 100 μl formamide, and analyzed by quantitative primer extension as described above. The $t_{1/2}$ for *polh* RNA was calculated using the values obtained for *polh* RNA content at each time point.

Nuclear run-on assays

Transcription rates were measured by nuclear run-on assays as described by Nevins (1987) and Ooi *et al.* (1989) with modifications. To detect *polh* transcription, a 382-bp *Hin*dIII–*Kpn*I fragment from the *polh* open reading frame was cloned into the *Hin*dIII and *Kpn*I sites of M13mp18 and M13mp19. Sense and antisense single-stranded probes were produced by standard methods (Sambrook *et al.,* 1989). To detect *vp39* transcription, a 400-bp *Hin*cII fragment from the *vp39* open reading frame was cloned into the *Hin*cII site of pBluescript(−). This plasmid, the single-stranded *polh* probes, and pUC18 were applied to nitrocellulose using an SRC-96 filter manifold (Schleicher and Schuell, Keene, NH).

Cells were seeded in T-150 flasks at a density of 1.8×10^7 cells per flask and infected with either AcMNPV-E2 or AcFPβgal at an m.o.i. of 10. At 24 hr p.i., medium was removed from the flasks and replaced with 7 ml of cell breakage buffer [10 m*M* Tris–HCl (pH 7.4), 140 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, 0.5% Nonidet-P40]. Detached cells from each flask were pelleted, resuspended in breakage buffer, and added back to the flasks. The flasks were rocked gently on ice for 5 min and nuclei were pelleted from the extracts by centrifugation at 1000 *g* for three min. The nuclei were washed once in breakage buffer and resuspended in 50 μl 2$\times$ transcription buffer [20 m*M* Tris–HCl (pH 7.4), 140 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* DTT, 20% glycerol]. The nuclear run-on transcription reaction was carried out immediately after preparation of the nuclei in

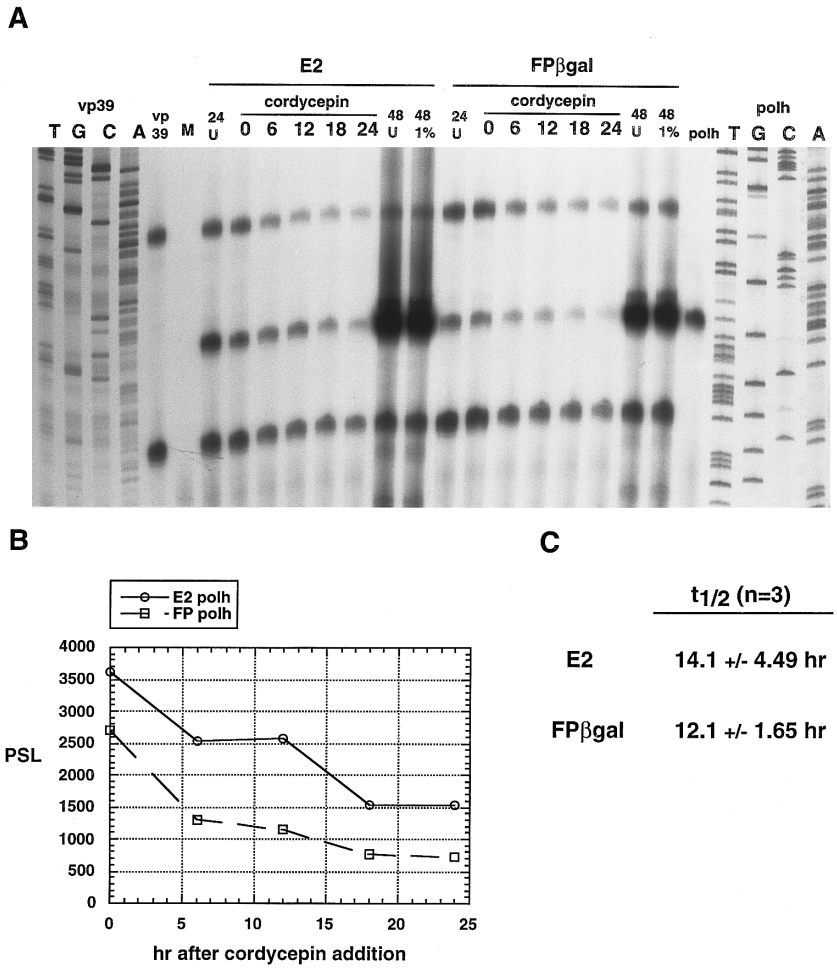

FIG. 5. Influence of *25K* on *polh* RNA stability. (A) Sf9 cells were infected with wild-type AcMNPV (E2) or AcFPβgal and treated with unaltered medium (U), medium containing 200 μg/ml cordycepin (cordycepin), or medium containing 1% ethanol (1%) beginning at 24 hr p.i. Cytoplasmic RNA samples were prepared at the time of cordycepin addition (0, 24 U, 24 1%) and every 6 hr thereafter for 24 hr, up to 48 hr p.i. (6, 12, 18, 24, 48 U, 48 1%). The quantity of *polh* and *vp39* RNA in each sample was determined by primer extension analysis using the *polh*- and *vp39*-specific primers as previously described. Control primer extension reactions on RNA from mock-infected (M) or AcMNPV-infected cells with the individual primers (*vp39, polh*) are shown, as are *vp39* and *polh* sequencing ladders. (B) The *polh* extension products obtained from cells infected with wild-type AcMNPV (E2, open circles) or FPβgal (open squares) were quantitated as previously described and graphed as a function of hours after cordycepin addition. *Polh* RNA quantity is represented as phosphorescence-stimulated luminescence units (PSL) read by the BAS2000 bio-imaging system. (C) Half-lives ($t_{1/2}$) of *polh* RNA were calculated from three separate experiments, with one standard deviation reported.

a 100-μl volume with 50 μl nuclei, 25 μl 3000 Ci/mmol [α-^{32}P]UTP, 1 μ*M* cold UTP, 0.66 m*M* ATP, CTP, and GTP, 10 m*M* creatine phosphate, 20 μg creatine kinase, and 1 m*M* DTT. Reactions were incubated at 28° for 30 min. Total RNA was prepared from the nuclei by the method of Chirgwin *et al.* (1979) and aliquots of the labeled RNA were hybridized to nitrocellulose filter-immobilized single-stranded and double-stranded DNA probes as described by Nevins (1987). The filters were incubated in 2× SSC (0.3 *M* sodium chloride, 0.03 *M* sodium citrate) containing 15 μg/ml RNase A for 30 min at 37° and then subjected to autoradiography. The radioactive signals on the filters were quantitated using the FUJIX BAS2000 bio-imaging analyzer system as previously described. This experiment was carried out three times to ensure the reproducibility of the results.

Analysis of IE-1 protein content

The quantity of IE-1 protein produced during viral infection was determined by radioimmunoprecipitation with polyclonal IE-1 antiserum (Kovacs *et al.,* 1992) that was preadsorbed with an equal volume of uninfected Sf9 cell extract. Sf9 cells were infected with AcMNPV-E2 or AcFP-βgal at an m.o.i. of 10 and metabolically labeled from 20 to 24 or from 44 to 48 hr p.i. with 250 μCi per milliliter of Translabel. At the end of the labeling period, the cells were pelleted and resuspended in 40 μl of 0.5× protein sample buffer [2% sodium dodecyl sulfate, 2% β-mercaptoethanol, 25 m*M* Tris–HCl (pH 6.8), 5% glycerol, 0.05% bromophenol blue]. After boiling and shearing of genomic DNA, the extracts were diluted 50× with extraction buffer [EB; 50 m*M* Tris–HCl (pH 8.0), 100 m*M* NaCl, 1%

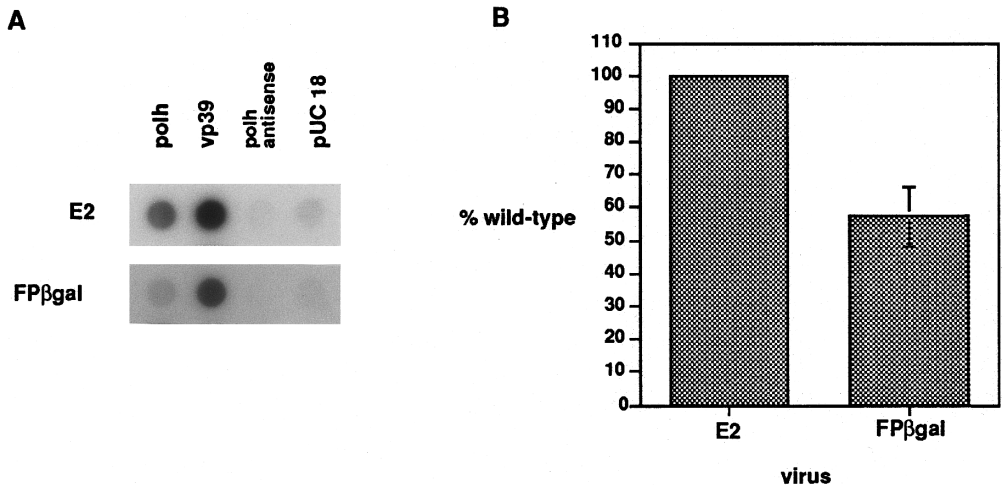

FIG. 6. Influence of *25K* on *polh* transcription rate. (A) The *polh* transcription rate in Sf9 cells infected with wild-type AcMNPV (E2) or AcFPβgal was determined by nuclear run-on assays. Total RNA from infected cell nuclei harvested at 24 hr p.i. was labeled by transcription in the presence of [α-^{32}P]UTP. The labeled RNA was then recovered from the nuclei and hybridized to a nitrocellulose filter-immobilized *polh*-sense probe (polh), a double-stranded *vp39* probe (vp39), a *polh*-antisense probe (polh antisense), or pUC18. (B) The hybridization signals from three separate experiments were quantitated with the BAS2000 bio-imaging system, and the *polh:vp39* ratios were calculated. The wild-type (E2) *polh:vp39* ratios were set at 100%, and the average percentage wild-type *polh:vp39* ratio for AcFPβgal was calculated and graphed, with one standard deviation reported.

NP-40], precleared with normal rabbit serum and fixed *Staphylococcus aureus* Cowan I (SAC), and immunoprecipitated with preadsorbed IE-1 antibody as previously described (Jarvis and Summers, 1989). Immunoprecipitates were analyzed by SDS–PAGE on 10% polyacrylamide gels. The gels were stained with Coomassie blue, dried, and subjected to autoradiography. Alternatively, proteins were transferred from the gels to PVDF membranes (Immobilon-P; Millipore, Bedford, MA) and analyzed by immunoblotting with a 1:1000 dilution of preadsorbed anti-IE-1. The immunoblots were autoradiographed to confirm the location of the IE-1 protein on the gels. Labeled IE-1 protein was quantitated with the FUJIX BAS2000 bio-imaging analyzer system as previously described.

RESULTS

Influence of *25K* on *polh* expression

The previous analysis of polyhedrin synthesis in *25K* mutant-infected cells described in Jarvis *et al.* (1992) was repeated and extended to include four spontaneously occurring *25K* mutants isolated by Beames and Summers (1989). The results showed a significant reduction in the accumulation of polyhedrin in the nuclei of *25K* mutant-infected cells from 20 to 24 hr p.i. (Fig. 2A). A minor reduction in nuclear amounts of polyhedrin accumulation was also evident in mutant-infected cells from 44 to 48 hr p.i. (Fig. 2B) These results were similar to those observed by Jarvis *et al.* (1992) for the AcFPβgal virus, showing that 25K is involved in the normal synthesis and nuclear localization of polyhedrin.

The presence of other minor protein species comigrating with polyhedrin in the cytoplasmic extracts and the contamination of nuclear polyhedrin gel slices with a protein migrating just above polyhedrin in the nuclear extract lanes prevented an accurate measurement of the relative quantities of polyhedrin protein synthesized by wild-type and mutant viruses in Fig. 2. To more precisely and accurately quantitate the influence of *25K* mutations on *polh* expression, recombinant viruses were produced in which *polh* in the *25K* mutants was replaced with a *polh*–CAT fusion gene (AcFPβgal-CAT, AcFP480-1-CAT, etc.). The levels of CAT activity produced in cells infected with these mutants and harvested at 24, 48, or 72 hr p.i. were generally reduced relative to wild-type by approximately 1.5- to 3-fold (Fig. 3). However, no statistically significant differences in CAT activity were observed at 48 and 72 hr p.i. between wild-type virus (Ac360CAT) and AcFP875-2-CAT. This result could be explained by the observation that AcFP875-2 (and therefore AcFP875-2-CAT) produces some full-length, wild-type 25K protein, albeit at lower levels than wild-type virus (Harrison and Summers, 1995b). No statistically significant differences in CAT activity were observed among the *25K* mutants at any of the time points examined.

To examine the molecular mechanism by which *25K* influences *polh* expression, quantitative primer extension analyses were used to compare the steady-state levels of *polh* RNA in wild-type- or *25K* mutant-infected cells (Fig. 4). Each reaction included both a *polh* primer and a primer for the baculovirus major capsid protein gene *vp39* (Thiem and Miller, 1989), with the latter serving as an internal standard. Quantitation of the signals from the primer extension products and normalization of the *polh* signals with respect to the *vp39* signals (Fig. 4B) con-

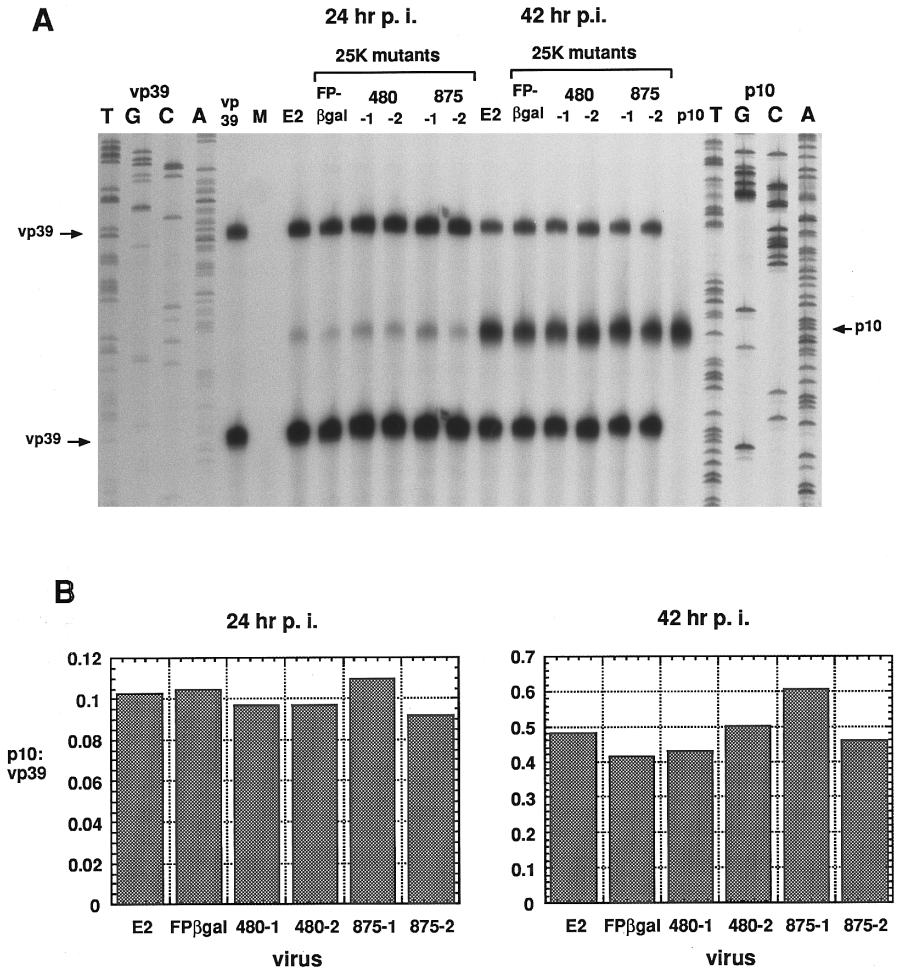

FIG. 7. Influence of *25K* on steady-state levels of *p10* RNA. (A) RNA samples from Sf9 cells infected with wild-type AcMNPV (E2) or various *25K* mutants were used for primer extension analyses with *p10*- and *vp39*-specific primers as previously described. Primer extension reactions with RNA from mock (M)- or AcMNPV (E2)-infected cells and the individual primers (vp39, p10) are shown, as are *vp39* and *p10* sequencing ladders. The locations of the *p10* and *vp39* extension products are indicated to the right and left of the autoradiograph, respectively. (B) The graphs show the ratios of *p10* to *vp39* RNA, calculated for each sample in (A) as previously described.

firmed that steady-state *polh* RNA levels were reduced in cells infected with all the FP mutants by two- to three-fold at 24 hr p.i. and $1\frac{1}{2}$- to 2-fold at 42 hr p.i.

25K mutations could affect *polh* RNA quantity by affecting RNA stability, transcription rate, or both. To distinguish among these possibilities, experiments were designed to compare the stability and rate of synthesis of *polh* RNA in wild-type AcMNPV- or *25K* mutant-infected cells. RNA stability was measured by infecting Sf9 cells with either wild-type AcMNPV or AcFPβgal and adding cordycepin at 24 hr p.i. to halt transcription. Turnover of *polh* RNA was measured by primer extension reactions on cytoplasmic RNA samples prepared every 6 hr after the addition of cordycepin (Figs. 5A and 5B). Three repetitions of this experiment showed that there was no statistically significant difference between the $t_{1/2}$ values calculated for wild-type and AcFPβgal *polh* transcripts (Fig. 5C). To examine the effect of *25K* mutation on *polh* transcription rate, nuclear run-on assays were carried out on nuclei harvested from wild-type- or AcFPβgal-infected cells at 24 hr p.i. (Fig. 6A). Quantitation of the *polh* and *vp39* signals indicated that the *polh:vp39* ratio was reduced by approximately twofold in AcFPβgal infections, indicating that the *polh* transcription rate was reduced by an amount consistent with the reduction in *polh* RNA levels and *polh* promoter-driven CAT expression (Fig. 6B). Together, these results indicate that 25K enhances polyhedrin synthesis by influencing the rate of *polh* transcription.

Influence of *25K* on *p10* expression

Since *polh* and *p10* are both classified as baculovirus very late genes, it was of interest to examine the influence of *25K* mutation on *p10* expression. Quantitative primer extension analyses were used to measure *p10* RNA levels at 24 and 42 hr p.i. The results of these analyses showed that there was no difference in the

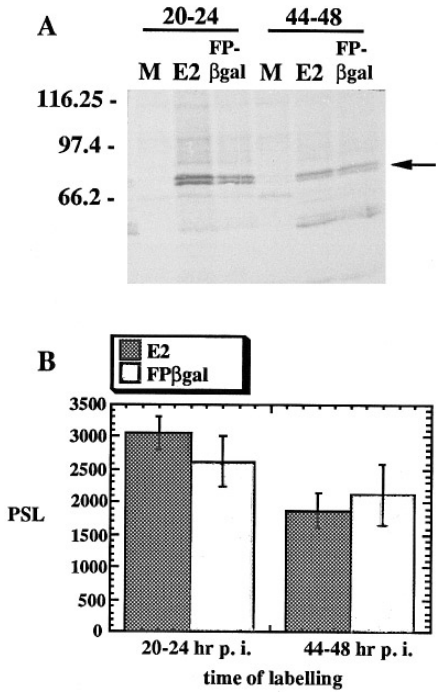

FIG. 8. Influence of *25K* on IE-1 protein synthesis. (A) Sf9 cells were mock-infected (M) or infected with wild-type AcMNPV (E2) or AcFPβgal and radiolabeled from 20–24 or 44–48 hr p.i. with Translabel. At the end of the labeling period, IE-1 was immunoprecipitated from total cell lysates with rabbit polyclonal IE-1 antiserum (Kovacs *et al.,* 1992). The positions of molecular weight markers are indicated to the left of the autoradiograph. (B) The amounts of radioactivity in each IE-1 band were quantitated using the FUJIX BAS2000 bio-imaging system and graphed. The graph shows the average amount of IE-1 from three replicate immunoprecipitation experiments with one standard deviation.

amount of *p10* RNA detected in wild-type- or mutant-infected cells (Fig. 7), indicating that *p10* expression was not affected by *25K* mutation. These results are consistent with the results of other studies which have shown that *polh* and *p10* expression are differentially regulated (Roelvink *et al.,* 1992; van Oers *et al.,* 1992; Chaabihi *et al.,* 1993).

Influence of *25K* on *ie-1* expression

The results of a previous study with an *ie-1* temperature-sensitive mutant (*ts*B821; Ribiero *et al.,* 1994) suggested that the AcMNPV IE-1 transregulatory protein is directly involved in the expression of *polh* (Choi and Guarino, 1995). Hence, it was possible that *25K* influences *polh* expression indirectly by influencing the expression of *ie-1* during the late phase of infection. To test this hypothesis, IE-1 protein production was compared in wild-type- and AcFPβgal-infected cells at 24 and 48 hr p.i. by both radio-immunoprecipitation (Fig. 8) and immunoblotting (data not shown). Neither technique revealed a difference in the amounts of IE-1 protein produced by wild-type AcMNPV or AcFPβgal.

These results were extended by examining *ie-1* promoter-driven CAT expression by wild-type and AcFPβgal viruses containing an *ie-1*/CAT fusion gene in place of *polh.* Cells were infected with these viruses and CAT activity was measured in cell lysates prepared at 24, 48, 72, and 96 hr p.i. (Fig. 9). No statistically significant difference in CAT activity was detected between the wild-type and the *25K* mutant versions of any of the four types of *ie-1*/CAT viruses produced. Together, these results indicated that 25K does not influence *polh* gene expression indirectly by modulating *ie-1* expression.

DISCUSSION

Two baculovirus genes (*vlf-1* and *ie-1*) have been identified which appear to specifically influence very late gene expression (McLachlin and Miller, 1994; Choi and Guarino, 1995). The results of this study indicate that 25K is also required for optimal expression of the *polh* gene. The 25K protein is required directly or indirectly for normal levels of *polh* transcription, as the *polh* transcription rate was significantly reduced in cells infected with the *25K* mutant AcFPβgal. In a recent study, Todd and coworkers (1996) used a transient expression assay to screen the AcMNPV genome for genes that are required specifically for very late gene expression. *25K* was not identified as a gene required for optimal activity of *polh* or *p10* promoters in this assay. However, it is possible that this type of assay may be limited in the identification of late genes involved in the regulation of very late gene expression, as these might not be expressed at a level in transient assays which is sufficient to have a significant effect on very late gene promoter-mediated expression.

If one defines a very late expression factor as being required for both *polh* and *p10* expression, then 25K cannot be classified as a very late expression factor because it does not have a detectable effect on *p10* expression. However, the difference in the effects of *25K* mutation on *polh* and *p10* expression is similar to previously reported differences in the regulation of these genes. Although the *polh* and *p10* promoters apparently have similar *cis* regulatory sequences, a study on *polh* and *p10* promoter-driven reporter gene expression revealed that the *p10* promoter is activated earlier than the *polh* promoter and that the maximum level of *p10* expression is lower than that of *polh* (Roelvink *et al.,* 1992). It also has been shown that deletion of the *polh* promoter either had no effect on *p10* promoter-driven expression (van Oers *et al.,* 1992) or increased *p10* RNA levels by a small amount (Chaabihi *et al.,* 1993), whereas deletion of the *p10* promoter increased *polh* protein and RNA production by three- to fourfold (Chaabihi *et al.,* 1993). These results suggest that the *polh* and *p10* promoters compete for at least one expression factor which is available in limited quantities and that the *p10* promoter is apparently less dependent on this factor(s) than the *polh* promoter. It is possible

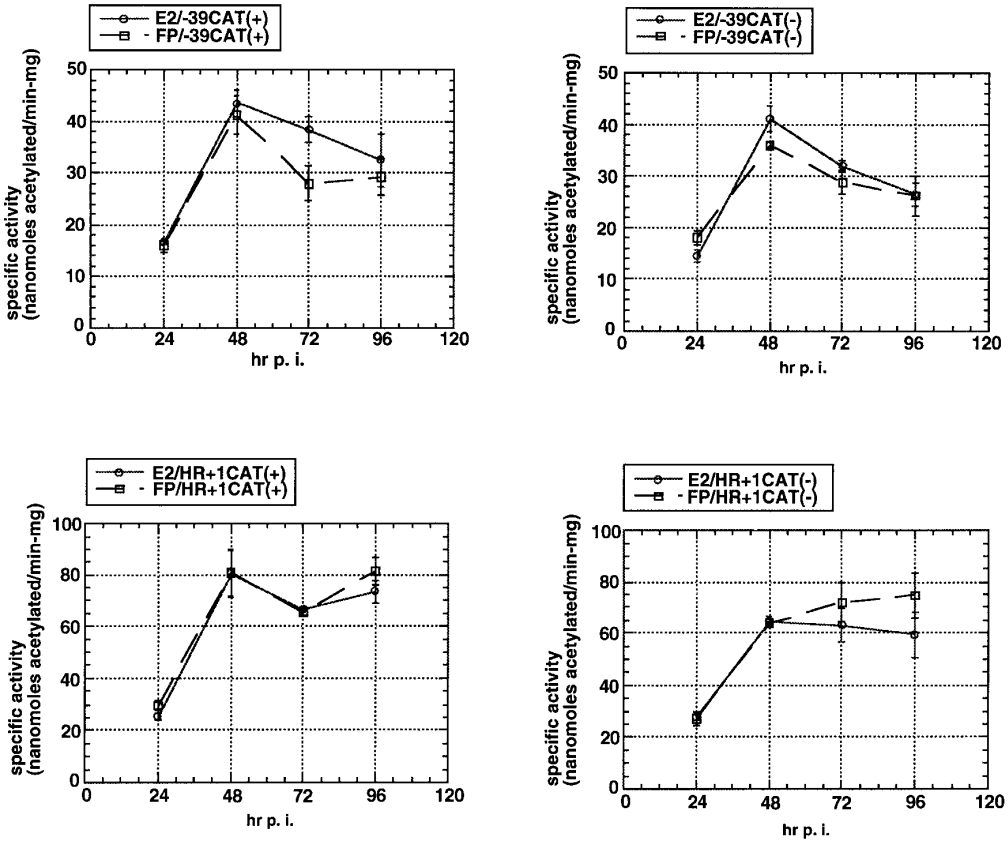

FIG. 9. Influence of *25K* on *ie-1* promoter-driven CAT gene expression. Sf9 cells were infected with recombinant baculoviruses containing an *ie-1*/CAT fusion gene in place of *polh* in a wild-type (E2/CAT) or mutant (FP/CAT) *25K* background. At 24, 48, 72, or 96 hr p.i., extracts from infected cells were prepared and assayed for CAT activity. Each graph shows the average specific activity in three replicate samples/virus/time point for each recombinant virus used in this experiment. The error bars represent one standard deviation.

that the 25K protein functions as a limiting expression factor for the *polh* gene, or it may influence the expression of such a factor. Finally, a *vlf-1* temperature-sensitive mutant (*ts*B837) has different effects on *polh* and *p10* RNA levels. At the nonpermissive temperature, *polh* RNA levels are sharply reduced, but *p10* RNA levels are reduced by only a moderate degree (McLachlin and Miller, 1994). Since the effects of *vlf-1* and *25K* mutation on *polh* and *p10* expression are similar, with *polh* RNA levels being more severely affected than *p10* RNA levels in both cases, it is possible that 25K and VLF-1 influence very late gene expression by a similar mechanism.

The role of the 25K protein in *polh* transcription at the molecular level is unknown. Immunogold labeling experiments with 25K antisera revealed that some 25K protein is detected in the nucleus of infected cells (Harrison and Summers, 1995b). This observation is consistent with the possibility that 25K could have a more direct effect on *polh* transcription. However, the 25K protein is also clearly associated with amorphous masses in the cytoplasm of infected cells (Harrison and Summers, 1995b). Hence, it is equally possible that this cytoplasmic 25K influences *polh* transcription indirectly, perhaps by influencing the expression (at a posttranscriptional level) of transcription factors. A possible mechanism not supported by the experiments of this study was that 25K influences *polh* expression indirectly by influencing *ie-1* expression. There is evidence that the transcription factor IE-1 participates directly in *polh* expression (Choi and Guarino, 1995), but neither IE-1 protein levels nor *ie-1* promoter-driven reporter gene expression were influenced by mutation of the *25K* gene. While this result indicates that 25K does not influence *polh* transcription through an effect on *ie-1* expression, it does not rule out the possibility that 25K functions by influencing the expression of *vlf-1* or other genes that are required for optimal levels of *polh* transcription.

Although mutations in the *25K* gene cause defects in occluded virus morphogenesis, in our previous study the *25K* gene product was not detected directly in association with occluded virus assembly events in the nucleus (Harrison and Summers, 1995b). Hence, the findings of Jarvis *et al.* (1992) and this study suggest that *25K* might indirectly influence virus assembly by influencing the expression of occluded virus-specific genes. The reduction in polyhedrin synthesis and nuclear localization associated with *25K* mutation could account for the reduced number of viral occlusions observed with infections by

FP mutants by resulting in a significantly decreased concentration of polyhedrin available in the nucleus for occlusion assembly. However, there was no difference in BV production or intranuclear ODV envelopment in cells infected with Ac360-CAT (which does not produce polyhedrin) or wild-type virus (AcMNPV-E2; Harrison and Summers, 1995a). Therefore, reduced *polh* expression and polyhedrin nuclear localization alone cannot explain the increase in BV production and the defects in ODV envelopment observed with *25K* mutants. It is possible that 25K is required for the normal expression of multiple viral genes involved in occlusion and ODV morphogenesis. The collective effect of impaired expression of these genes in *25K* mutant-infected cells could account for the FP phenotype. Future experiments will be designed to identify other viral genes affected by *25K* mutation and to determine the mechanism by which 25K affects gene expression.

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